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13. ABSTRACT (Maximum 200 Words) Endomorphins, endogenous brain opiates with the highest affinity and specificity for the mu opiate receptor, potently produce analgesia. A rapid brain-to-blood efflux system could give misleading results when entry rates are determined. Preliminary results show that endomorphin-1 and endomorphin-2 are saturably transported from brain to blood, as shown by self-inhibition by an excess of that peptide. There also was cross-inhibition of each endomorphin by the other, indicating shared components for the efflux system. CGRP, substance P, or constriction of the sciatic nerve did not decrease efflux. Furthermore, chronic pain induced by sciatic nerve constriction caused a striking decrease in endomorphin-2 immunoreactivity on the nerve-injured side in the spinal cord. Preliminary results also indicate that immunoreactive substance P, but not CGRP, was modestly reduced on the injured side, showing that immunoreactive endomorphin-2 in the spinal cord is decreased during the development of chronic pain. We also found a dissociation of analgesic and rewarding effects of endomorphin-1 in rats. This could indicate that the potent effects of endomorphin-1 on pain might not always be associated with the addictive properties of reward.				
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INTRODUCTION:

The main purpose of this research is to examine the crossing of the blood-brain barrier (BBB) by the endomorphins. Endomorphin-1 and endomorphin-2 have been isolated by us from human and bovine brain (12,25). They represent the brain opiates with the highest affinity and specificity for the mu opiate receptor, which is the site of most of morphine's actions. They produce analgesia in mice with a potency similar to that of morphine. We are studying how their passage across the BBB is influenced by pain and its modulating peptides.

BODY:

Determination of the rate of entry of substances across the BBB involves measurements of radioactivity in the brain with suitable considerations of degradation and binding to the luminal surface of the endothelial cells of the capillaries that comprise the BBB. The counts in the brain would give misleading results if the substance being injected in blood were transported out of the brain faster than the usual rate of the reabsorption of cerebrospinal fluid (CSF).

Very few of the peptides tested for entry into brain have saturable efflux systems. We have tested the following substances for efflux systems, but not found any: insulin (10), mahogany(1377-1428) (17), melanin-concentrating hormone (15), leptin (7,20), CART(55-102) (16), orexin A (14), neuropeptide Y (13), pancreatic polypeptide (3), cycloHis-Pro (4), β -amyloid(1-28) (5), epidermal growth factor (21), and agouti-related protein(83-132) (17), interleukin (IL)-1 α and -1 β (5), TNF- α (11), IL-6 (7), IL-2 (23), brain-derived neurotrophic factor (15), and leukemia-inhibitory factor (15). However, there is an efflux system across the BBB for Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH₂), an opiate modulating tetrapeptide (1).

The brain-to-blood efflux for intact Tyr-MIF-1 is stereospecific (9) and does not transport any of the peptide fragments contained within the tetrapeptide (6). Removal of only a single hydroxyl group from the N-terminal amino acid prevents transport (6), yet it can transport the pentapeptide opiate Met-enkephalin, which has a much different structure (8).

Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH₂) differs from Tyr-MIF-1 by one amino acid (5), and these peptides are differentially metabolized in different areas of the brain (4). Tyr-W-MIF-1 can be transported by the same Peptide Transport System (PTS-1) (2) as Tyr-MIF-1, but less robustly, raising the possibility that there might be a separate system for it or related peptides (11).

Endomorphin-1 (Tyr-Pro-Trp-Phe-NH₂) differs from Tyr-W-MIF-1 by a single amino acid (25). It is not known whether endomorphin-1, or the related endomorphin-2 (Tyr-Pro-Phe-Phe-NH₂), is saturably transported from brain to blood. Therefore, we quantified the brain-to-blood transport of endomorphin-1 and endomorphin-2 across the BBB and examined the influence of pain modulation by sciatic nerve constriction as well as by calcitonin gene-related peptide (CGRP) and substance P.

To do this, about 25,000 cpm of ¹²⁵I-endomorphin-1 or ¹²⁵I-endomorphin-2, purified by HPLC with mean specific activity about 2100 Ci/mmol, was injected into the brain of anesthetized mice at a site 1 mm lateral and 0.2 mm posterior to the bregma with a Hamilton syringe (25). It has been

shown by autoradiography that by this method with these coordinates, material is accurately delivered to the lateral ventricle of the mouse brain (20). Binding studies showed that these iodinated endomorphins were biologically active.

The animals were studied at 0, 2, 5, 10, and 20 min after injection. The 0 min value was determined in mice overdosed with anesthesia before injection (15). The half-time disappearance was determined from the regression line obtained from the plot of the logarithm of brain radioactivity against time.

Inhibition with unlabeled endomorphin-1, endomorphin-2, and Tyr-MIF-1 was tested at a dose of 10 nmol ($\sim 5 \mu\text{g}/\text{mouse}$), as was the supernatant of a saturated solution of cyclosporin injected together with the tracer amount of radiolabeled endomorphins. Cross-inhibition with Tyr-W-MIF-1, CGRP, and substance P was tested at $5 \mu\text{g}/\text{mouse}$.

Sciatic nerve constriction involved exposure of the common sciatic nerve in anesthetized mice. Proximal to the bifurcation, one tight ligature of 4-0 chromic gut was tied around one-half to one-third the diameter of the nerve. This nerve-injury paradigm produces significant hyperalgesia, which peaks at 14 days (19). Four groups of about 6 mice each were tested 14 days after receiving ^{125}I -endomorphin-1 or ^{125}I -endomorphin-2 with and without sciatic nerve constriction.

For each part of the experiment, groups were compared by analysis of variance (ANOVA) followed by Duncan's multiple comparisons test. Regression lines were determined by the least squares method and the differences between slopes compared by GraphPad Prism statistical software (GraphPad Software Inc, San Diego, CA).

The preliminary results show that endomorphin-1 and endomorphin-2 are saturably transported from brain to blood. This was established by self-inhibition of the transport of each radiolabeled peptide by an excess of that peptide.

There also was cross-inhibition of the rate of efflux of each endomorphin by the other: an excess of endomorphin-2 inhibited the efflux of endomorphin-1, and an excess of endomorphin-1 inhibited the efflux of endomorphin-2. This indicates shared components for the brain-to-blood transport of these endogenous opiate peptides. After incubation of the endomorphins in fresh human CSF for 2 h, much longer than the duration of the present experiments, preliminary results from another study show at least 85% of the endomorphins remaining intact, consistent with the negligible degradation of Tyr-MIF-1 in rat CSF (18).

The rates of efflux of endomorphin-1 and endomorphin-2 were not cross-inhibited by Tyr-MIF-1, indicating that the endomorphins are not transported by PTS-1. Tyr-W-MIF-1 is transported by PTS-1 even though it differs from Tyr-MIF-1 by one of its four amino acids (11); yet Tyr-W-MIF-1, also differing from the tetrapeptide endomorphin-1 by only a single amino acid, did not affect the transport of the endomorphins.

CGRP and substance P, two larger peptides involved in the transmission of painful stimuli, did not decrease the brain-to-blood transport of either endomorphin-1 or endomorphin-2 under the

conditions of the experiment. Similarly, constriction of the sciatic nerve did not alter the rate of efflux when tested two weeks later. The apparent lack of effect of cyclosporin, moreover, indicates that the efflux of the endomorphins does not occur by the P-glycoprotein system (22).

Thus, even though saturable efflux out of the brain for endogenous peptides and polypeptides seems to be relatively uncommon, we found that endomorphin-1 and endomorphin-2 leave the brain by a shared transport mechanism. These results demonstrate the existence of a new transport system across the BBB which saturably transports endomorphins from brain-to-blood.

Endomorphin-2 has been localized in the spinal cord (12) where it is involved in primary sensory afferent fibers (20). It is not known whether endomorphin-2 is changed by pain. We induced chronic pain by the same method used above - sciatic nerve constriction. Two weeks later, the time of maximal hyperalgesia, we found a striking decrease in endomorphin-2 immunoreactivity on the nerve-injured side as compared with either the control side or control mice. The change was restricted to the medial dorsal horn in the lumbar segments enervated by the sciatic nerve.

Preliminary results indicate that substance P immunoreactivity was modestly reduced in the lumbar sections of the injured side at that time; CGRP immunoreactivity, however, did not seem to be changed, suggesting that non-specific deficits resulting from the injury probably don't explain the changes in immunoreactive endomorphin-2. Thus, this study shows for the first time that immunoreactivity of an opiate peptide - in this case, endomorphin-2 - is decreased during the development of chronic pain.

Another study showed a dissociation of analgesic and rewarding effects of endomorphin-1 in rats (24). If corroborated by further studies at different doses, this could indicate that the potent effects of endomorphin-1 on pain might not be associated with the addictive properties of reward. This paper is the only one of the studies described above that is published (or in press yet). It is enclosed in the appendix.

KEY RESEARCH ACCOMPLISHMENTS

1. The first demonstration of a shared saturable brain-to-blood transport system for endomorphin-1 and endomorphin-2, not influenced by CGRP or substance P.
2. The first demonstration that reduction of an endogenous opiate peptide (endomorphin-2) in primary afferents of the spinal cord is associated with injury-induced chronic pain. Substance P, but not CGRP, was modestly affected.
3. The first demonstration of an endogenous mu opiate producing potent analgesia in the absence of reward behavior.

REPORTABLE OUTCOMES

1. Kastin AJ, Fasold MB, Smith RR, Horner KA, Zadina JE. Saturable brain-to-blood transport of endomorphins. *Exp. Brain Res.* In press, 2001.

2. Smith RR, Martin-Schild S, Kastin AJ, Zadina JE. Decreases in endomorphin-2-like immunoreactivity concomitant with chronic pain after chronic constriction injury. *Neuroscience*. In press, 2001.
3. Wilson AM, Soignier RD, Zadina JE, Kastin AJ, Nores WL, Olson RD, Olson GA. Dissociation of analgesic and rewarding effects of endomorphin-1 in rats. *Peptides* 21:1871-1874, 2000.

CONCLUSIONS

A saturable brain-to-blood transport system exists for the endomorphins and is shared by both of them. This could speculatively be interpreted to indicate that the endomorphins are such powerful opiates that a mechanism needs to exist to prevent too high levels from occurring in the brain. The lack of effect of CGRP and substance P on this transport indicates that their effects in the opposite direction will not be complicated by the efflux system.

The decrease in immunoreactive endomorphin-2 in the spinal cord, associated with thermal hyperalgesia shown by decreased paw-withdrawal, indicates the loss of an inhibitory influence on pain transmission, helping to explain the actions of endogenous opiates on pain.

The dissociation of rewarding and analgesic effects of endomorphin-1 has possible clinical significance in that pain reduction might occur without the reward potential for abuse.

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APPENDIX

Attached reprint of reported outcome 3.

Dissociation of analgesic and rewarding effects of endomorphin-1 in rats

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Abstract

The μ -receptor is the primary mediator of the effects of morphine and the endogenous opiates, endomorphin-1 and endomorphin-2. Here we demonstrate a dissociation of the analgesic and rewarding effects of endomorphin-1 in rats. Tail-flick results revealed that endomorphin-1 produced significant analgesic effects within 10-min after injection. However, it failed to show reward properties in the standard 45-min conditioned place preference (CPP) paradigm or in an abbreviated 10-min pairing which paralleled the time frame of the tail-flick findings. Morphine induced both analgesia and reward. Endomorphin-1 therefore is the first mu opiate shown to produce potent analgesia in the absence of reward behavior, and thus may have significant clinical potential. © 2000 Elsevier Science Inc. All rights reserved.

1. Introduction

The three opiate receptors, designated δ , κ , and μ , found in the peripheral and central nervous systems, mediate the biologic functions of opiate peptides. The μ -receptor, which is the main target of potent analgesics such as morphine [4,12], is considered to be the primary factor in the development of drug addiction, because of its central role in the mediation of reward. Endogenous ligands have been discovered for the δ - and κ -receptors (enkephalins and dynorphins, respectively), but a specific and selective ligand for the μ -receptor remained elusive until recently. Isolated from bovine [15] and human [3] brain tissue, endomorphin-1 and endomorphin-2 are considered endogenous ligands for the μ -receptor because they have the highest known affinity, specificity, and biopotency for this receptor [2,6,15] and they are anatomically positioned to activate it [7,16].

A prominent characteristic of μ -opiate agonists is their ability to elicit strong rewarding effects, as evidenced in the

conditioned place preference (CPP) test [9]. The CPP paradigm consists of pairing a desirable stimulus with one distinctive environment and a neutral stimulus with another contrasting environment and then testing drug-free animals for their preference of environments. A number of studies including previous studies in this laboratory [10] have established that morphine induces CPP. Although the analgesic effects of endomorphins were determined to be equipotent with morphine and of prolonged duration in mice [15], the rewarding properties of these peptides were previously unknown.

2. Method

2.1. Animals

Male Sprague-Dawley rats weighing 275–455 g were obtained from Harlan-Sprague Dawley (Indianapolis, IN) and were housed in accordance with APA guidelines [1] on a 12-h light/dark cycle with food and water available ad libitum. All procedures were approved by the institutional animal care and use committee.

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2.2. Surgery

The rats were anesthetized with ketamine/xylazine (80 mg/kg/8 mg/kg IM) and placed into a Kopf stereotaxic frame with the incisor bar positioned at 3.5 mm below the interaural line. A scalp incision was made and three small holes drilled into the skull. According to the coordinate system provided by Paxinos and Watson [11], a 23-gauge stainless steel guide cannula was positioned at 0.9 mm posterior to the coronal suture, 1.2 mm lateral to the sagittal suture, and 2.6 mm ventral to the dorsal surface of the skull. The guide cannula was attached to the skull with two stainless steel jeweler's screws and cranioplastic cement (Plastics One, Roanoke, VA, USA). To prevent occlusion, a dummy cannula was inserted into the guide cannula. The rats were allowed at least 7 days of postsurgery recovery before the start of behavioral testing. On each of the last four days of the recovery period, the rats were individually handled for about 3 min. At the completion of behavioral testing, anesthetized rats were injected with 5 μ l methylene blue dye, which was used to verify the accurate placement of the cannulae.

2.2.1. Drug administration. Microinjections were delivered into the right lateral ventricle with a 10 μ l Hamilton syringe connected by PE10 tubing to a 30-gauge injection cannula cut to protrude 1.9 mm beyond the tip of the guide cannula. The coded solutions of 10 μ g of each drug were injected in a volume of 5 μ l over a 60-sec period. To reduce reflux and to allow for diffusion, the injection cannula was left in place for an additional 30 sec.

2.3. Conditioned place preference apparatus and procedure

The CPP apparatus consisted of three compartments made of wood, with clear Plexiglas tops to permit observations. The two treatment compartments were identical in size (45 \times 45 \times 30 cm) but varied according to visual and tactile cues. One compartment was painted black, with a wire-mesh floor. The other compartment was painted white, with a wood-chip floor. The treatment compartments were separated by a wooden partition and interconnected by a third compartment (36 \times 25 \times 24 cm), which was painted gray, with a smooth wooden floor. This third compartment served as the start box during the testing trials. During conditioning trials, a wooden partition was inserted to block the entrance to the start box.

The CPP procedure consisted of six conditioning trials and one test trial. The six conditioning trials alternated between three pairings of the assigned experimental drug and three saline pairings, with one trial completed per day. Immediately after the saline or experimental injections, the animal was confined to the appropriate treatment compartment for 45 min in experiment 1 and for 10 min in experiment 2. On test day, uninjected rats were placed in the start

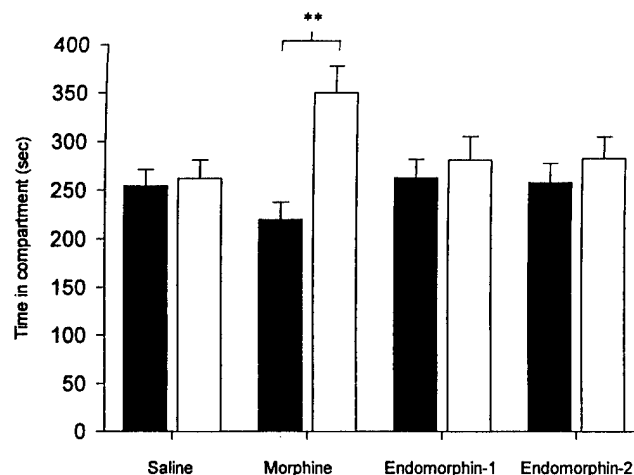


Fig. 1. Effects of icv administration (10 μ g/5 μ l) of morphine ($n = 16$), endomorphin-1 ($n = 15$), endomorphin-2 ($n = 16$) and saline ($n = 17$) in the CPP test. Bars represent the mean (\pm SEM) seconds spent in the saline- (black bars) and drug-paired (white bars) compartments. ** Indicates significant difference between saline and drug treatments ($P < .01$).

box and tested for preference. The time spent in each compartment was recorded for 15 min with stopwatches (one for each treatment compartment). The rats were considered to have entered a compartment when both forelimbs touched the floor of that compartment.

2.4. Tail-flick procedure

Three to four days after the completion of CPP testing, the analgesic effect of the compounds was assessed with the focused-light tail-flick test (Columbus Instruments, Columbus, OH). These measurements were determined by placement of the tip of the tail across a slit through which light from a focused bulb projected. The intensity of the heat stimulus was adjusted so that the baseline latency was about 7 sec. The time between the onset of the light and the abrupt movement of the tail was the measured response. Each session consisted of 4 latency determinations at 0, 5, 10, and 20 min after each ICV microinjection. To minimize tissue damage, a 20-sec cut-off latency was employed.

3. Results

Fig. 1 summarizes the CPP data, illustrating the rewarding properties of morphine. A 4 \times 2 \times 2 \times 2 (Treatment \times Compartment \times Order \times Side) mixed factorial ANOVA was performed, with side serving as the repeated variable, yielding a significant two-way (Treatment \times Side) interaction ($F(3,48) = 5.50$, $P < 0.01$, $\eta^2 = .26$, power = .92), indicating that the time spent in the drug-paired compartment versus the saline-paired compartment was not equivalent for every drug. Simple effects analysis of treatment revealed that only morphine induced CPP ($F(1,30) =$

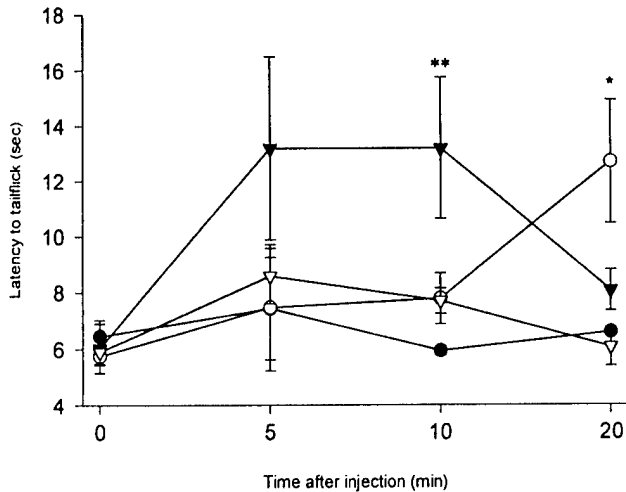


Fig. 2. Analgesic effects of icv administration ($10 \mu\text{g}/5 \mu\text{l}$) of morphine (○, $n = 7$), endomorphin-1 (▼, $n = 4$), endomorphin-2 (▽, $n = 5$), and vehicle (●, $n = 5$) in the tail-flick test 3–4 days after completion of the CPP test. Data are expressed as mean (\pm SEM) latency to tail-flick in seconds for each group. *Indicates significant difference from saline control (* $P < 0.05$, ** $P < .01$).

26.73, $P < 0.01$, $\eta^2 = .47$, power = .99). No other significant results were obtained.

During early CPP data collection, endomorphin-1 and endomorphin-2 were clearly not producing a place preference effect, so we added an assessment of the analgesic actions of all the peptides using the focused-light tail-flick test on the remainder of the animals in the study, to ensure their potency. Fig. 2 shows that endomorphin-1 induced short-lasting analgesia that reached maximum efficacy 10 min after injection ($F(3,17) = 6.14$, $P < 0.01$, $\eta^2 = .52$, power = .91). Endomorphin-2 did not exhibit any significant analgesic effects at the dose tested using rats in this paradigm. As expected, morphine produced maximum analgesia 20 min after administration ($F(3,17) = 4.29$, $P < 0.01$, $\eta^2 = .43$, power = .77). The time course of analgesia suggested that the length of the conditioning trials in the initial CPP test might have been a critical factor for the absence of a rewarding effect. If the effect of the endomorphins dissipated so rapidly, the majority of the 45-min of CPP might be equivalent to the saline condition. It was also possible that the animals experienced withdrawal symptoms after the short-lasting analgesic effects disappeared, producing a negative affect, since the endomorphins are relatively new endogenous opiates whose effects with regard to tolerance and dependence are not well understood.

We determined that a second CPP test with a 10-min conditioning trial was necessary to more fully assess the rewarding properties of endomorphin-1 during its optimum analgesic effects. The results of this second evaluation of the CPP test confirmed our original finding and are summarized in Fig. 3. Endomorphin-1 did not produce a preference effect during the time of maximum analgesia, demonstrating a dissociation of these properties, previously unknown

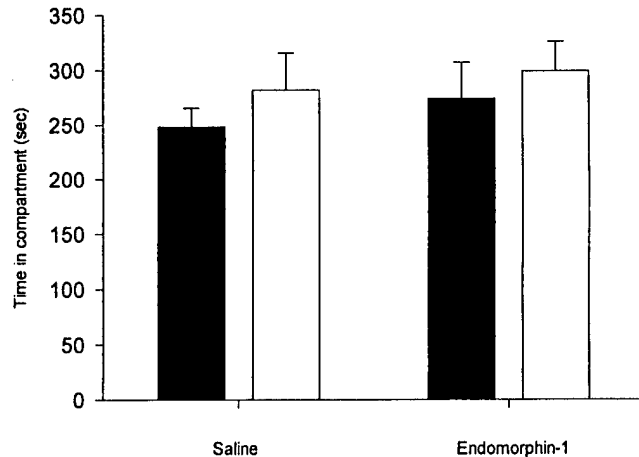


Fig. 3. Effects of icv administration ($10 \mu\text{g}/5 \mu\text{l}$) of endomorphin-1 ($n = 15$) and vehicle ($n = 14$) in the CPP test with 10-min trials. Bars represent the mean (\pm SEM) seconds spent in the saline- (black bars) and drug-paired (white bars) compartments. No significant differences were obtained.

for μ -receptor agonists. Morphine, however, does induce CPP with conditioning trials of this short duration [9], indicating that the paradigm can work with only 10-min trials.

4. Discussion

The primary finding of this paper is the behavioral dissociation of analgesia and reward functions for endomorphin-1. It is the first demonstration among the opiates of attenuation of pain, as measured by the tail-flick test, without accompanying pleasurable effects in the CPP paradigm. One possible explanation for this unprecedented finding might be that the rewarding properties of opiates are mediated through δ -, as well as μ -opiate receptors. Because morphine can activate δ as well as μ receptors, although with lower affinity, its rewarding properties could reflect actions at both sites. Endomorphin-1, by contrast, has much higher selectivity for the μ receptor [15], and is therefore unlikely to have concerted effects on reward through both μ and δ receptors. However, the rewarding effects of morphine have been reported to be eliminated in μ -receptor knockout mice [8], indicating that the μ receptor is sufficient to account for morphine's rewarding properties. Growing evidence supports the idea that different μ agonists can interact differentially with the μ receptor [13] and induce differential activation of μ -receptor mediated cellular events [14].

The results of the present study therefore could reflect differential agonist actions at the μ receptor for endomorphin-1 and morphine. It also is possible that endomorphin-1 has rewarding and CPP-inducing properties at higher doses than that used here. Nevertheless, the present results clearly demonstrate that the analgesic effects of endomorphin-1 can be dissociated from its rewarding effects. This suggests possible clinical significance for endomorphin-1, because

the induction of analgesia without reward would reduce the potential for abuse. It is therefore possible that endomorphin-1 or its analogs may lead to the development of newer analgesics with less addictive properties than the traditional opiate agonists [5].

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